Genetically Diverse Filoviruses in *Rousettus* and *Eonycteris* spp. Bats, China, 2009 and 2015

Xing-Lou Yang,[1] Yun-Zhi Zhang,[1] Ren-Di Jiang,[1] Hua Guo, Wei Zhang, Bei Li, Ning Wang, Li Wang, Cecilia Waruhiu, Ji-Hua Zhou, Shi-Yue Li, Peter Daszak, Lin-Fa Wang, Zheng-Li Shi

Genetically divergent filoviruses detected in *Rousettus* and *Eonycteris* spp. bats in China exhibited 61%–99% nt identity with reported filoviruses, based on partial replicase sequences, and they demonstrated lung tropism. Co-infection with 4 different filoviruses was found in 1 bat. These results demonstrate that fruit bats are key reservoirs of filoviruses.

Filoviruses (family *Filoviridae*) are nonsegmented, negative-strand RNA viruses belonging to 3 genera: *Marburgvirus*, *Ebolavirus*, and *Cuevavirus*. *Marburgvirus* comprises 1 species, *Marburg marburgvirus,* which includes Marburg virus (MARV) and Ravn virus. *Ebolavirus* comprises 5 species, *Zaire ebolavirus* (ZEBOV), *Sudan ebolavirus*, *Bundibugyo ebolavirus*, *Taï Forest ebolavirus*, and *Reston virus* (RESTV). *Cuevavirus* comprises 1 species, *Lloviu cuevavirus* (*1*). Filovirus-associated diseases, especially those caused by ZEBOV and MARV, are recognized as a major threat to public health, causing high rates of death among humans and nonhuman primates.

Bats have been implicated as natural reservoirs for filoviruses (*2,3*) on the basis of serologic evidence from 19 bat species in 8 countries across Asia, Africa, and Europe *(2,4–9)*. In addition, filovirus RNA has been detected in 8 bat species from 7 countries in the same regions (*2–4,10–13*). Outbreaks of Marburg hemorrhagic fever among miners in Uganda in 2007 were traced to bat MARV (*11*). In addition, we previously discovered filovirus antibodies in several bat species in China (*14*). This finding was further confirmed by He et al., who detected filovirus RNA in brown fruit bats (*Rousettus leschenaultii*) in China (*10*). Considering the diversity of bat species in the world, long-term surveillance of bat filoviruses is essential for better understanding of distribution, diversity, and ecology of these viruses. We conducted a study to determine the diversity of filoviruses among bats in Yunnan Province, China.

The Study

We captured 150 apparently healthy adult bats from 2 caves in Yunnan Province, China: 1 in Jinghong City in November 2009, and 1 in Mengla County in December 2015 (Table 1; Figure 1). The bat species we collected were *Hipposideros armiger*, *Aselliscus stoliczkanus*, *Myotis ricketti*, *Rhinolophus Monoceros*, *Miniopterus fuscus*, *Ia io*, *Eonycteris spelaea*, and *Rousettus* sp. We humanely killed all bats and collected their hearts, intestines, lungs, spleens, kidneys, livers, brains, and blood for testing. We used 2 methods to analyze bat lung tissues for presence of filovirus RNA: first, we used nested PCR with the primers FV F1/R1 and FV F2/R2 (*10*), and next, we used quantitative PCR (qPCR) with 3 groups of qPCR with primers and probes designed from viral sequences obtained in this study (online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/23/3/16-1119-Techapp1.pdf).

Using degenerate nested PCR, we detected filovirus RNA in 15 fruit bat specimens (*E. spelaea* and *Rousettus* sp.); the specimens comprised 10 (23.3%) of 43 *E. spelaea* and *Rousettus* sp. collected in 2009 and 5 (11.9%) of 42 collected in 2015. Using qPCR, we detected filovirus RNA in 20 specimens from *E. spelaea* (n = 4) and *Rousettus* sp. (n = 16) bats: 10 (23.3%) of the bats were collected in 2009 and 10 (23.8%) in 2015. No filovirus was detected in other bat species studied (Table 1). The 310-bp L gene sequences (GenBank accession nos. KX371873–KX371890) exhibited 65%–99% nt identity among themselves and 61%–99% nt identity with known filoviruses. Phylogenetic analysis showed that the sequences from the bats formed 3 independent groups, groups 1–3. Groups 1 and 2 comprised 6 and 11 sequences, respectively, all of which were obtained in this study (Figure 2). Group 3 comprised 2 sequences, 1 from this study and 1 previously published (*10*). Pairwise distance analysis indicated that sequences in group 1 share the highest nucleotide identity (75%–78%) with MARV and those in group 2 share the highest identity (69%–74%) with Ravn virus. The 2 sequences in group 3 are highly similar and share 66%–70% nt identity with other filovirus species. Of note, 1 bat specimen (no. 9447) was co-infected

Author affiliations: Chinese Academy of Sciences, Wuhan, China (X.-L. Yang, R.-D. Jiang, H. Guo, W. Zhang, B. Li, N. Wang, L. Wang, C. Waruhiu, Z.-L. Shi); Dali University, Dali, China (Y.-Z. Zhang); University of Chinese Academy of Sciences, Beijing, China (R.-D Jiang, H. Guo, N. Wang); Yunnan Institute of Endemic Diseases Control and Prevention, Dali (Y.-Z. Zhang, J.-H. Zhou); Wuhan University, Wuhan (S.-Y. Li); EcoHealth Alliance, New York, New York, USA (P. Daszak); Duke–NUS Graduate Medical School, Singapore (L.-F. Wang)

DOI: http://dx.doi.org/10.3201/eid2303.161119

[1]These authors contributed equally to this article.

Table 1. Filovirus infection detected in bat samples by PCR, ELISA, and Western blot, Yunnan Province, China, 2009 and 2015*

Bat species, by year and month of collection/location	No. positive/no. tested (%)					
		Quantitative	ELISA†		Western blot†	
	RT-PCR	PCR	ZEBOV	RESTV	ZEBOV	RESTV
2009 Nov/Jinghong City						
Hipposideros armiger	0/15	0/15	0/15	0/15	0/15	0/15
Rhinolophus monoceros	0/4	0/4	0/4	0/4	0/4	0/4
Ia io	0/3	0/3	0/3	0/3	0/3	0/3
Miniopterus fuscus	0/1	0/1	0/1	0/1	0/1	0/1
Myotis ricketti	0/27	0/27	1/27 (3.7)	1/27 (3.7)	1/27 (3.7)	1/27 (3.7)
Eonycteris spelaea and *Rousettus* sp.	10/43 (23.3)	10/43 (23.3)	5/43 (11.6)	6/43 (13.9)	2/43 (4.6)	2/43 (4.6)
2015 Dec/Mengla County						
Aselliscus stoliczkanus	0/15	0/15	0/15	0/15	0/15	0/15
E. spelaea and *Rousettus* sp.	5/42 (11.9)	10/42 (23.8)	14/25 (56)	7/25 (28)	11/25 (44)	4/25 (16)

*EBOV, *Zaire ebolavirus*; RESTV, *Reston virus*.
†ELISA and Western blot results for samples collected in 2009 were from a previous study (*14*).

with 4 different filovirus strains (BtFiloYN9447–1 to 9447–4) with high divergence (Figure 2; online Technical Appendix Figure 1). To further determine the phylogenetic relationship of these viruses with known filoviruses, we amplified more L gene sequence (1,475 bp) for strains BtFiloYN2162 and BtFiloYN9447–1. Similar to the 310-bp sequences, the 1,475-bp sequence of the BtfiloYN2162 shared 99% identity with BtDH04 at the nucleotide level, the 1,475-bp sequence of BtFiloYN9447–1 shared 62%–71% with known filoviruses.

To determine the tissue tropism of these viruses, we performed qPCR with primers and probes designed for each of the 3 different groups (online Technical Appendix Table). Results showed that filoviruses were mainly located in the lung and that genome copy numbers ranged from 29 to 523,582/mg of tissue (Table 2). Only 2 bat blood samples (nos. 2202 and 9447) were positive for filovirus RNA; 5 samples (nos. 2202, 2188, 9434, 9442, and 9447) contained filoviruses with more widespread tissue tropism. We were unable to isolate virus from PCR-positive samples by using Vero-E6 cells.

To detect filovirus IgG and IgM, we expressed His-tagged truncated nucleoproteins from RESTV or ZEBOV in *Escherichia coli* and used them as antigens (online

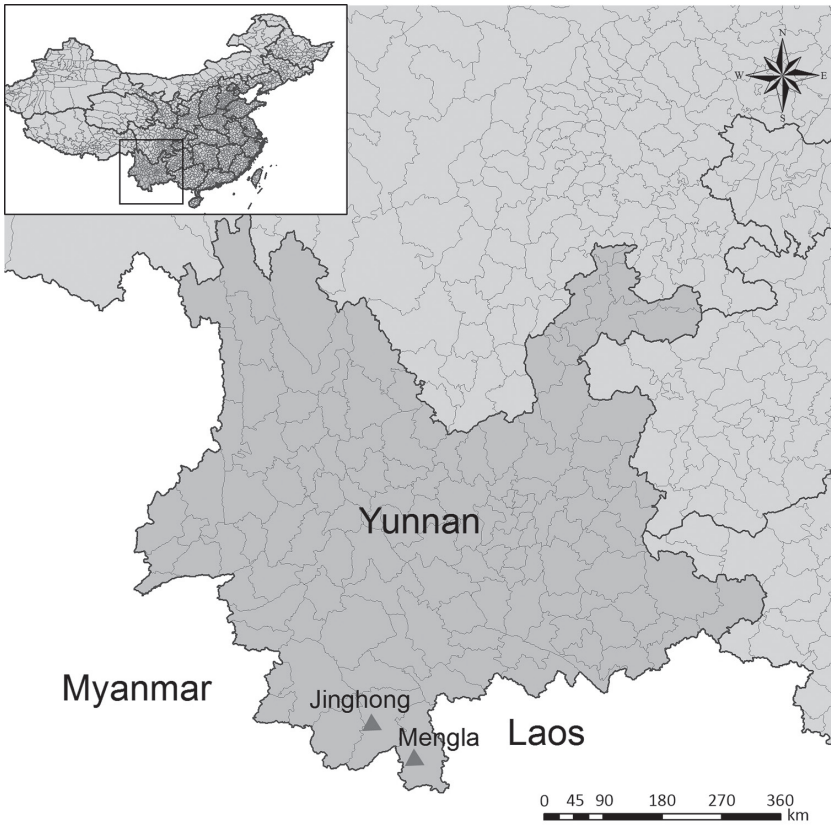


Figure 1. Bat collection sites for a study on genetically diverse filoviruses in *Rousettus* and *Eonycteris* spp. bats in China. Triangles indicate Jinghong City and Mengla County, Yunnan Province, where 150 apparently healthy adult bats were collected from 2 caves in November 2009 (Jinghong City) and December 2015 (Mengla County). Inset map shows the location of Yunnan Province in China.

Table 2. Virus tropism and quantification in different tissues of *Eonycteris spelaea* and *Rousettus* sp. bats, China, 2009 and 2015

Sample no.	Species	Sex	Primer group*	Positive organs (viral genome copies/mg tissue or viral genome copies/μL blood)
2162	*E. spelaea*	M	3	Lung (119)
2176	*Rousettus* sp.	M	2	Lung (2,103)
2180	*Rousettus* sp.	F	2	Lung (42)
2181	*Rousettus* sp.	M	2	Lung (202)
2187	*Rousettus* sp.	F	1	Lung (46)
2188	*Rousettus* sp.	F	1	Lung (43), liver (400), kidney (42)
2190	*Rousettus* sp.	M	2	Lung (195)
2196	*Rousettus* sp.	F	1	Lung (123)
2199	*Rousettus* sp.	F	1	Lung (74)
2202	*Rousettus* sp.	F	1	Lung (864), liver (368), kidney (342), intestine (254), heart (807), blood (1)
9428	*Rousettus* sp.	F	2	Lung (156)
9434	*Rousettus* sp.	F	1	Lung (554), spleen (3,014), kidney (380), heart (320), intestine (2751)
9435	*Rousettus* sp.	F	2	Lung (127)
9442	*E. spelaea*	M	2	Lung (180), spleen (88)
9445	*Rousettus* sp.	M	1	Lung (143)
9447	*Rousettus* sp.	F	2	Lung (132), liver (154), spleen (661)
9447	Rousettus sp.	F	1	Lung (106,606), liver (220,051), spleen (523,582), kidney (41,653), brain (4,885), heart (17,982), intestine (11,788), blood (485)
9454	*Rousettus* sp.	M	1	Lung (448)
9457	*E. spelaea*	M	1	Lung (52)
9459	*Rousettus* sp.	F	2	Lung (182)
9463	*E. spelaea*	M	2	Lung (114)

*These represent primers and probes designed based on partial sequences of the virus L gene obtained in this study. Sequence information is provided in the online Technical Appendix (https://wwwnc.cdc.gov/EID/article/23/3/16-1119-Techapp1.pdf).

Technical Appendix). In this experiment, we used 25 bat samples from 2015 that had enough serum volume for testing; 14 samples showed a strong cross-reaction with the ZEBOV nucleoprotein, and among them, 7 showed a weak cross-reaction with RESTV nucleoprotein. We used Western blotting to confirm these results; 11 of the 25 samples were positive for ZEBOV nucleoprotein and 4 for RESTV nucleoprotein (Table 1; online Technical Appendix Figure 2). No samples overlapped between those identified as positive by PCR and those identified as positive by serologic testing. Results of a serum neutralization assay with HIV pseudovirus carrying the ZEBOV glycoprotein showed that the ELISA-positive samples had no cross-neutralization activity to the pseudovirus (*14*).

Conclusions

We detected novel filovirus sequences with high divergence in *E. spelaea* and *Rousettus* sp. bats in China. Phylogenetic analysis of partial sequences suggested that at least 3 distinct groups of filovirus are circulating in fruit bats in China. The distances between these sequences indicates that the 3 groups may represent 3 novel species or genera. Of interest, we detected antibodies reacting more strongly to ZEBOV than RESTV nucleoprotein in some filovirus RNA–negative samples, suggesting that the bats were infected with another/other filovirus(es) cross-reactive with ZEBOV nucleoprotein or that nucleoproteins of the novel filoviruses were cross-reactive with ZEBOV and RESTV nucleoproteins.

The bat samples in this study were collected from 2 caves in 2009 and 2015, respectively; the caves are ≈200 km metric apart. Across the 2 different years and locations, we detected closely related viruses and found 1 bat that was acutely co-infected by 4 different filoviruses; this finding suggests that these viruses have been circulating in the 2 bat species and that densely populated bat caves provide opportunity for cross-infection with different viruses. However, considering the migration ability of the fruit bat, we cannot exclude the possibility that there are exchanges of virus between the bats in these two caves. Longitudinal surveillance with tracking tags may help to better understand the spatial–temporal distribution of these viruses in bat populations.

In previous reports, filoviruses were primarily detected in liver and spleen tissues (*4,15*). In our study, we primarily detected filoviruses in the lung. We suspect that lung tissues are the major target for these bat filoviruses. Thus, these filoviruses may have the potential to be transmitted through the respiratory tract.

These results will be helpful in providing a better understanding of the distribution and diversity of filoviruses, which may have implications for public health. Considering their feeding habitats, fruit bats are often in close contact with domestic animals and human populations. It is therefore necessary to establish long-term and proactive surveillance of these viruses and related diseases.

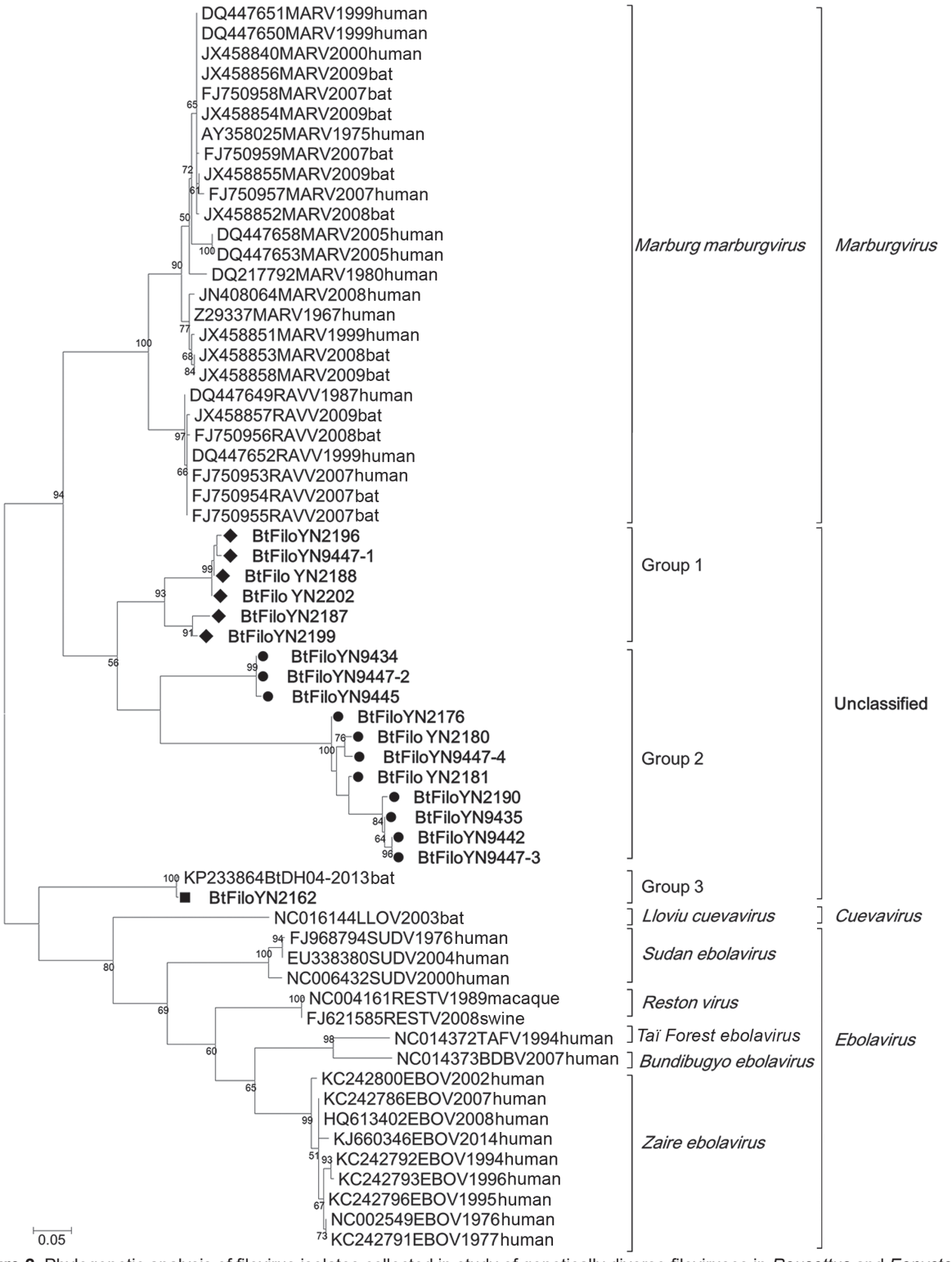


Figure 2. Phylogenetic analysis of filovirus isolates collected in study of genetically diverse filoviruses in *Rousettus* and *Eonycteris* spp. bats in China, compared with reference isolates. Analysis was based on a 310-bp segment of the filovirus L gene. Bootstrap values lower than 50 are not shown. The maximum-likelihood tree was constructed based on the 310-bp segment with 1,000 bootstrap replicates. The sequences obtained in this study are marked with a triangle (group 1), black dot (group 2), or rectangle [group 3). Sequences from GenBank are listed by their accession numbers, followed by the virus name, collection year, and host. Scale bar indicates nucleotide substitutions per site.

This work was funded by a joint National Natural Science Foundation of China grant (81290341) and the China Mega-Project for Infectious Disease grant (2014ZX10004001-003) from the Minister of Science and Technology of the People's Republic of China (to Z-L.S.); a Scientific and Technological Basis Special Project grant (2013FY113500; to Z-L.S. and Y-Z.Z.); a National Natural Science Foundation of China grant (81260437) and State Key Laboratory for Infectious Diseases Prevention and Control grant (2013SKLID302) (to Y-Z.Z.); and a United States Agency for International Development Emerging Pandemic Threats PREDICT project grant (cooperative agreement AID-OAA-A-14-00102; to P.D.D.).

Dr. Yang is as a research assistant at Wuhan Institute of Virology, Chinese Academy of Sciences. His primary research interests include viral epidemiology and viral characterization of small mammals.

References

1. King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, editors. Virus taxonomy: ninth report of the International Committee on Taxonomy of Viruses. San Diego (CA): Academic Press; 2012.
2. Olival KJ, Hayman DT. Filoviruses in bats: current knowledge and future directions. Viruses. 2014;6:1759–88. http://dx.doi.org/10.3390/v6041759
3. Jayme SI, Field HE, de Jong C, Olival KJ, Marsh G, Tagtag AM, et al. Molecular evidence of Ebola Reston virus infection in Philippine bats. Virol J. 2015;12:107. http://dx.doi.org/10.1186/s12985-015-0331-3
4. Leroy EM, Kumulungui B, Pourrut X, Rouquet P, Hassanin A, Yaba P, et al. Fruit bats as reservoirs of Ebola virus. Nature. 2005;438:575–6. http://dx.doi.org/10.1038/438575a
5. Swanepoel R, Smit SB, Rollin PE, Formenty P, Leman PA, Kemp A, et al.; International Scientific and Technical Committee for Marburg Hemorrhagic Fever Control in the Democratic Republic of Congo. Studies of reservoir hosts for Marburg virus. Emerg Infect Dis. 2007;13:1847–51. http://dx.doi.org/10.3201/eid1312.071115
6. Kuzmin IV, Niezgoda M, Franka R, Agwanda B, Markotter W, Breiman RF, et al. Marburg virus in fruit bat, Kenya. Emerg Infect Dis. 2010;16:352–4. http://dx.doi.org/10.3201/eid1602.091269
7. Taniguchi S, Watanabe S, Masangkay JS, Omatsu T, Ikegami T, Alviola P, et al. Reston Ebolavirus antibodies in bats, the Philippines. Emerg Infect Dis. 2011;17:1559–60.
8. Olival KJ, Islam A, Yu M, Anthony SJ, Epstein JH, Khan SA, et al. Ebola virus antibodies in fruit bats, Bangladesh. Emerg Infect Dis. 2013;19:270–3. http://dx.doi.org/10.3201/eid1902.120524
9. Towner JS, Pourrut X, Albariño CG, Nkogue CN, Bird BH, Grard G, et al. Marburg virus infection detected in a common African bat. PLoS One. 2007;2:e764. http://dx.doi.org/10.1371/journal.pone.0000764
10. He B, Feng Y, Zhang H, Xu L, Yang W, Zhang Y, et al. Filovirus RNA in Fruit Bats, China. Emerg Infect Dis. 2015;21:1675–7. http://dx.doi.org/10.3201/eid2109.150260
11. Towner JS, Amman BR, Sealy TK, Carroll SA, Comer JA, Kemp A, et al. Isolation of genetically diverse Marburg viruses from Egyptian fruit bats. PLoS Pathog. 2009;5:e1000536. http://dx.doi.org/10.1371/journal.ppat.1000536
12. Negredo A, Palacios G, Vázquez-Morón S, González F, Dopazo H, Molero F, et al. Discovery of an ebolavirus-like filovirus in Europe. PLoS Pathog. 2011;7:e1002304. http://dx.doi.org/10.1371/journal.ppat.1002304
13. Amman BR, Carroll SA, Reed ZD, Sealy TK, Balinandi S, Swanepoel R, et al. Seasonal pulses of Marburg virus circulation in juvenile *Rousettus aegyptiacus* bats coincide with periods of increased risk of human infection. PLoS Pathog. 2012;8:e1002877. http://dx.doi.org/10.1371/journal.ppat.1002877
14. Yuan J, Zhang Y, Li J, Zhang Y, Wang LF, Shi Z. Serological evidence of ebolavirus infection in bats, China. Virol J. 2012;9:236. http://dx.doi.org/10.1186/1743-422X-9-236
15. Brauburger K, Hume AJ, Mühlberger E, Olejnik J. Forty-five years of Marburg virus research. Viruses. 2012;4:1878–927. http://dx.doi.org/10.3390/v4101878

Address for correspondence: Zheng-Li Shi, Key Laboratory of Special Pathogens and Biosafety, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China; email: zlshi@wh.iov.cn

Article DOI: http://dx.doi.org/10.3201/eid2303.161119

Genetically Diverse Filoviruses in *Rousettus* and *Eonycteris* spp. Bats, China, 2009 and 2015

Technical Appendix

Sample Collection

Bats were trapped by mist net in Jinghong (N21°46′17,” E101°30′22,” H882 m) and Mengla (N22°26′16,” E100°41′10,” H1300 m) in Yunnan Province, China, in November 2009 and December 2015. All bats were humanely sacrificed with isoflurane and their blood and tissue samples (hearts, intestines, spleens, lungs, kidneys, livers, and brains) were immediately collected. To minimize the influence to bat colony, juvenile bats were not included in the sampling. All the samples were transported to the laboratory and stored at –80°C until use. All sampling processes were performed by veterinarians with approval from the Animal Ethics Committee of the Wuhan Institute of Virology (Animal ethics approval number: WIVA05201202) and Yunnan Institute of Endemic Diseases Control and Prevention (Animal ethics approval number: 200801 and 201302).

RNA Extraction, Filovirus Detection, and Sequencing

Bat tissue samples (≈0.1 g) were cut into small pieces and homogenized in 1 mL of Minimum Essential Medium (MEM, GIBCO) using two steel beads and shaking on a Tissue Lyser II (Qiagen). RNA was extracted from 200 μL of supernatant of ground tissues with a High Pure Viral RNA Kit (Roche) following the manufacturer’s instructions. RNA was eluted in 50 μL elution buffer and then aliquoted and stored at –80°C. Nested RT-PCR using degenerate

primers (FV-F1/R1 and FV-F2/R2) for filovirus L gene was performed to detect filovirus sequences (*1*). A first round of PCR was performed in a 25-μL reaction mix containing 12.5 μL PCR 2× reaction mix buffer, 10 pmol of each primer, 2.5 mmol/L MgSO4, 20 U RNase inhibitor, 1 μL SuperScript III/ Platinum Taq Enzyme Mix, and 5 μL RNA. The PCR program was performed as follows: 50°C for 30 min and 94°C for 4 min; 36 cycles at 94°C for 30 sec, 52°C for 30 sec, and 68°C for 40 sec; and a final extension at 68°C for 5 min. The second round of PCR was done in a 25-μL reaction mix containing 2.5 μL PCR 10× reaction buffer, 10 pmol of each primer, 50 mmol/L $MgCl_2$, 0.5 mmol/L dNTPs, 0.1 μL Platinum Taq Enzyme (Invitrogen), and 1 μL first-round PCR product. The amplification procedure was performed as follows: 94°C for 5 min; 36 cycles at 94°C for 30 sec, 52°C for 30 sec, and 72°C for 40 sec; and a final extension at 72°C for 5 min. The amplicons were gel purified and sequenced directly using the ABI prism sequencer or cloned using pGEM-T Easy Vector System for sequencing if the direct sequencing failed (Technical Appendix Figure 1).

In vitro RNA Transcription and Establishment of Standard Curve

Based on the sequences obtained in this study, we designed 3 sets of primers and probes that specifically target the three groups of filovirus detected in China (Technical Appendix Table). Probes were labeled at the 5′ end with 6-carboxyfluorescein (6-FAM) and at the 3′ end with Black Hole Quencher 1 (BHQ1). Primers targeting fragments of templates from each of the 3 groups were synthesized (Technical Appendix Table). The forward primer contained a 5′-T7 RNA polymerase promoter sequence (TAATACGACTCACTATAGGG) to facilitate in vitro transcription. The template DNA was amplified from positive samples and transcribed into RNA with a MAXIscript Kit (Applied Biosystems), according to the manufacturer's instructions. The RNA transcripts were diluted with a 10-fold serial dilution and used as standards to calculate viral genome copy number.

Viral RNA Quantification

We performed qRT-PCRs with AgPath-ID One-Step RT-PCR Kit (Applied Biosystems) according to the manufacturer's instructions. Each 25-μL reaction mixture contained 12.5 μL of 2× RT-PCR buffer, 1 μL RT-PCR buffer enzyme mix, 400 nmol/L each primer, 120 nmol/L probe, and 3 μL of RNA extract. Amplification was carried out in 96-well plates with Step One PCR instructions (Applied Biosystems). Thermocycling conditions were as follows: 10 min at 45°C for reverse transcription; 10 min at 95°C for activation of Taq DNA polymerase; and 40 cycles at 95°C for 15 sec, 54°C for 20 sec, and 68°C for 20 sec. Each run included 3 viral positive template controls and 2 negative controls to monitor performance. Positive samples were characterized by a well-defined exponential fluorescence curve that crossed the cycle threshold (C_t) within 36 cycles. Specimens with a C_t >36 were repeated to exclude operational faults. Viral genome copy number was calculated in each sample using the standard curves of the template RNA.

Antibody Detection by ELISA

The nucleocapsid (NP) gene of RESTV and EBOV were synthesized based on reference sequences with reference numbers FJ621583 and L11365, respectively. Predicted epitope of NP were cloned to pPET28a (Sequence will be provided upon request). The His-tagged truncated NP of RESTV (Reston-NP) or EBOV (Zaire-NP) were expressed in *Escherichia coli* BL21 and purified with a His.Bind Kit (Novagen) following the manufacturer's instructions. The purified proteins strongly reacted to hyperimmune rabbit sera raised against the full-length NP protein of RESTV or EBOV by ELISA (*2*), and no cross reactive with each other. Purified truncated NPs from EBOV or RESTV were coated on ELISA plates at ≈100 ng/well, and bat sera were tested in triplicate at a dilution of 1:100, followed by detection with horseradish peroxidase (HRP)-conjugated protein A/G (Pierce) at a dilution of 1:20,000. Samples with a mean optical density at least 3-fold higher than that of the negative control were considered positive. A positive sample

and a negative sample from our previous results were used as a positive and negative control, respectively (*2*).

Antibody Detection by Western Blotting

Truncated EBOV or RESTV NPs (500 ng) were separated through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto a membrane made of polyvinylidene difluoride, which was activated with methanol. The membrane was then blocked with 3% bovine serum albumin in phosphate buffered saline for 1 h at 37°C. After blocking, the membrane was incubated with bat serum at a dilution of 1:100 overnight at 4°C. Rabbit serum immunized with truncated EBOV or RESTV NP was used as the positive control. The membrane was incubated with HRP-conjugated protein A/G (Pierce) at 1:40,000. The HRP substrate (Millipore) was added onto the blot and detected under chemiluminescent camera (Technical Appendix Figure 2). The positive serum samples were further confirmed by neutralization assay with pseudovirus with EBOV glycoprotein (GP) as previously reported (*2*).

References

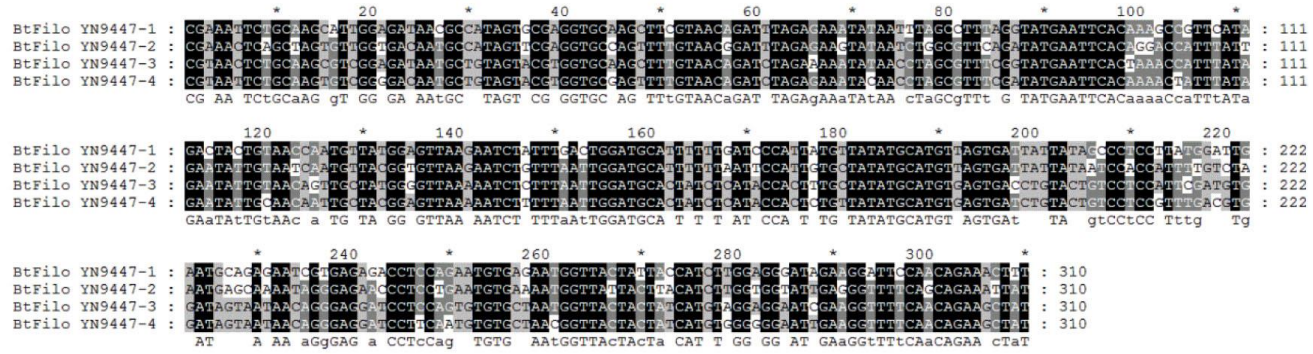
1. He B, Feng Y, Zhang H, Xu L, Yang W, Zhang Y, et al. Filovirus RNA in fruit bats, China. Emerg Infect Dis. 2015;21:1675–7. PubMed http://dx.doi.org/10.3201/eid2109.150260

2. Yuan J, Zhang Y, Li J, Zhang Y, Wang LF, Shi Z. Serological evidence of ebolavirus infection in bats, China. Virol J. 2012;9:236. PubMed http://dx.doi.org/10.1186/1743-422X-9-236

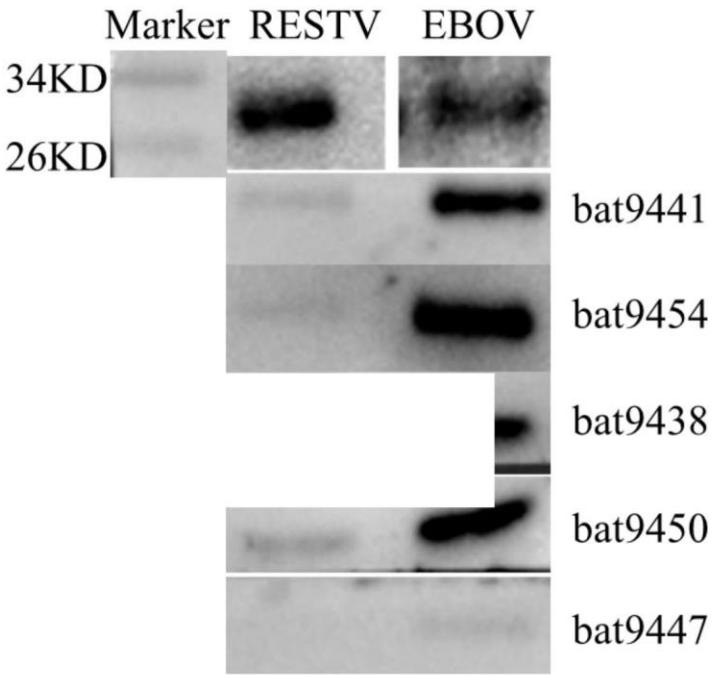
Technical Appendix Table. Primer sequences used for template preparation and viral quantification

Primer	Sequence*
Group1-Te-F	*TAATACGACTCACTATAGGG* ATCAAGCGTCGTGGCATCG
Group1-Te-R	ACAGGAGATGCAGGTCCAAAG
Group2-Te-F	*TAATACGACTCACTATAGG* ATGCAGGTCCATAGCTTC
Group2-Te-R	TCATCAAGCGTCGTGGCAT
Group3-Te-F	*TAATACGACTCACTATAGG* GCATCAAGCGTCATGGCA
Group3-Te-R	CTCTACTCCTCCTAAGTGAC
Group1-Q-F	TGATCCCATTATGTTATATGCAT
Group1-Q-R	TCCTTCTATCCCTCCAAGATG
Group1-Q-Probe	Fam-TAGTGATTATTATAGCCCTCCTT-BHQ1
Group2-Q-F	CCCACATGATAGTAGTAACC
Group2-Q-R	TGAGTGACCTGTACTGTCCTC
Group2-Q-Probe	Fam-TTAGCACACATTGGAGGATC-BHQ1
Group3-Q-F	ACAATCCACCTCACTGTCTAA
Group3-Q-R	CTCTACTCCTCCTAAGTGAC
Group3-Q-Probe	Fam-AAGCCAAAATCGAGAACATCCA-BHQ1

*The T7 promoter sequence is italicized.



Technical Appendix Figure 1. Alignment of different strains of filovirus YN9447.



Technical Appendix figure 2. Confirmation of positive serum samples by Western blotting. Truncated EBOV- or RESTV-NPs (500 ng) were transferred onto a polyvinylidene fluoride membrane. The membrane was incubated with bat serum at a dilution of 1:100 and detected with HRP-conjugated protein A/G (Pierce) at 1:40,000. Polyclonal antibodies against the full-length NP of RESTV or EBOV were used as positive controls.